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Review

# Hypoxia-Inducible Factors and the Response to Hypoxic Stress

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Oxygen ($O_2$) is an essential nutrient that serves as a key substrate in cellular metabolism and bioenergetics. In a variety of physiological and pathological states, organisms encounter insufficient $O_2$ availability, or hypoxia. In order to cope with this stress, evolutionarily conserved responses are engaged. In mammals, the primary transcriptional response to hypoxic stress is mediated by the hypoxia-inducible factors (HIFs). While canonically regulated by prolyl hydroxylase domain-containing enzymes (PHDs), the HIFα subunits are intricately responsive to numerous other factors, including factor-inhibiting HIF1α (FIH1), sirtuins, and metabolites. These transcription factors function in normal tissue homeostasis and impinge on critical aspects of disease progression and recovery. Insights from basic HIF biology are being translated into pharmaceuticals targeting the HIF pathway.

## Introduction

Aerobic organisms require oxygen ($O_2$) to produce energy. For this reason, $O_2$ deprivation creates significant stress in living cells. $O_2$ deprivation is also paradoxically linked to the inappropriate accumulation of free radicals, which cause additional stress on proteins and DNA in the cell. During low $O_2$ (hypoxic) conditions, therefore, cells activate a number of adaptive responses to match $O_2$ supply with metabolic, bioenergetic, and redox demands. Cells temporarily arrest in the cell cycle, reduce energy consumption, and secrete survival and proangiogenic factors. These events are coordinated by various cellular pathways, including the unfolded protein response (UPR), mTOR signaling, and gene regulation by hypoxia-inducible factors (HIFs). Initially identified as a regulator of erythropoietin (EPO) production, HIF is recognized as a key modulator of the transcriptional response to hypoxic stress. Besides its adaptive function in cellular stress responses, recent work has also revealed important roles for HIF in both physiological and pathological processes.

HIFs are obligate heterodimers consisting of an $O_2$-labile α subunit and a stable β subunit. Mammals possess three isoforms of HIFα, of which HIF1α and HIF2α (also known as EPAS1) are the most structurally similar and best characterized. HIF3α (or IPAS) exists as multiple splice variants, some of which inhibit HIF1α and HIF2α activity in a dominant-negative fashion (reviewed in Kaelin and Ratcliffe, 2008). HIF1α is expressed ubiquitously in all cells, whereas HIF2α and HIF3α are selectively expressed in certain tissues, including vascular endothelial cells (ECs), type II pneumocytes, renal interstitial cells, liver parenchymal cells, and cells of the myeloid lineage (reviewed in Bertout et al., 2008). In Figure 1, we highlight some of the novel mechanisms of HIFα regulation and HIFα transcriptional activity.

HIFα subunits heterodimerize with the stable HIF1β, or ARNT, subunit through their HLH and PAS domains. HIF heterodimers recognize and bind to hypoxia response elements (HREs) in the genome, which are similar to enhancer box (E box) motifs and have the consensus sequence G/ACGTG. Genome-wide chromatin immunoprecipitation experiments indicate that the correlation between HRE occupancy and hypoxic gene induction ranges from high for HIF1α-upregulated genes to low for both HIF2α-induced genes and HIF-repressed genes (Mole et al., 2009; Xia et al., 2009). In these latter cases, flanking sequences and additional regulatory elements appear to further specify HIF binding and target gene regulation. Recent examples of additional modulators of HIF-dependent gene regulation include the forkhead transcription factor FOXA2 and the chromatin modifier Reptin (Qi et al., 2010; Lee et al., 2010).

In this review, we will focus on recent insights into HIF expression, activation, and function in various cellular and tissue stress responses. We will also summarize emerging data on the crosstalk between HIFs and other metabolic regulators and provide a survey of recent pharmacologic strategies to modulate HIF activity in the treatment of diseases.

## HIFα Regulation by $O_2$ Availability

In well-oxygenated environments, HIFα subunits are hydroxylated at conserved proline residues. These modifications are mediated by PHDs, whose activities are regulated by $O_2$ availability (reviewed in Kaelin and Ratcliffe, 2008). Hydroxylated HIFα is, in turn, recognized and marked for proteosomal destruction by an E3 ubiquitin ligase, the von Hippel-Lindau protein (pVHL) complex. In the setting of hypoxic stress, PHD activity is diminished, and stabilized HIFα proteins can induce transcription of genes with adaptive functions.

### *PHD Regulation by $O_2$ and Metabolites*

Because of their dependence on $O_2$ as a direct substrate, PHDs have been proposed to be "oxygen sensors" linking cellular $O_2$ concentration to HIF molecular responses. This notion is

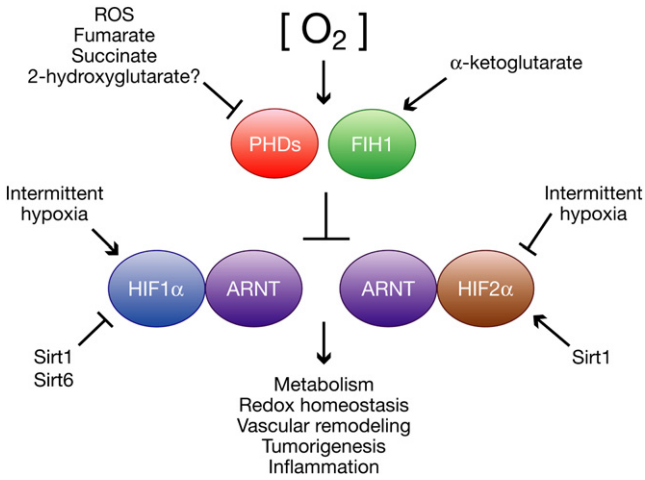


**Figure 1. Regulation and Function of HIFα Subunits**
In the presence of oxygen and α-ketoglutarate, HIF PHDs and FIH1 hydroxylate and inactivate HIFα. PHD and FIH1 activity are inhibited by hypoxia and certain intracellular metabolites, including ROS, fumarate, succinate, and potentially 2-hydroxyglutarate, resulting in HIFα stabilization. HIFα expression and/or activity is also regulated posttranslationally by sirtuins and IH. HIFα stabilization results in the activation of a transcriptional program with effects on metabolism, redox homeostasis, vascular remodeling, tumorigenesis, inflammation, and other processes.

supported by measurements of the apparent $K_M$ of PHD enzymes in vitro (reviewed in Kaelin and Ratcliffe, 2008). The $K_M$ values for oxygen were considerably higher than intracellular $pO_2$, suggesting that PHDs may function below saturation kinetics in vivo. While the enzymatic properties of PHDs in vitro do not necessarily reflect their properties in vivo, these measurements imply that $O_2$ availability can influence PHD activity across the entire physiological range.

Cellular metabolites can also influence PHD activity. PHDs utilize the tricarboxylic acid (TCA) cycle intermediate 2-oxoglutarate (α-ketoglutarate) as a substrate and can be inhibited by other TCA intermediates (Kaelin and Ratcliffe, 2008; Klimova and Chandel, 2008; Sudarshan et al., 2009). As we will describe later, several types of cancer have been shown to exploit this metabolic regulation and bear mutations that are likely to stimulate HIF activity.

### $O_2$-Dependent Regulation of HIFα by FIH1

HIFα subunits are also substrates for an asparaginyl hydroxylase: factor-inhibiting HIF-1α (FIH1). This enzyme is $O_2$ dependent and, thus, represents another component of the oxygen-sensing machinery. Hydroxylation by FIH1 disrupts a critical interaction between HIFα and coactivators p300/CBP, impairing HIF transcriptional activity (reviewed in Webb et al., 2009a; Mahon et al., 2001). However, FIH1 has other targets (Webb et al., 2009a, 2009b), indicating it may have HIF-independent functions as well.

*Fih1*$^{-/-}$ mice were recently generated (Zhang et al., 2010) and found to be viable, unlike *Vhl*$^{-/-}$ and *Phd2*$^{-/-}$ animals (Gnarra et al., 1997; Takeda et al., 2006). Mutant adult mice displayed a range of metabolic phenotypes including decreased weight, decreased adiposity, hyperventilation, and increased insulin sensitivity. Remarkably, several of these phenotypes were recapitulated by neuronal-specific deletion of *Fih1*. Moreover, when placed on a high-fat diet, *Fih1*$^{-/-}$ mice were less likely to develop insulin resistance, weight gain, and hepatic steatosis (or fatty liver). In correlation with these phenotypes, the authors observed that *Fih1* deletion affected the activity of AMPK, PGC1α, and PPARγ—factors which have been implicated in metabolism and metabolic diseases (Muoio and Koves, 2007; Towler and Hardie, 2007; Picard and Auwerx, 2002). Collectively, these data indicate that FIH1 regulates fat storage, insulin sensitivity, and organism growth. Furthermore, FIH1 deficiency can prevent features of obesity, diabetes, and nonalcoholic fatty liver disease (Adams and Lindor, 2007) in animals with increased caloric intake. Thus, FIH1 represents a novel drug target for treating these diseases.

Although several in vitro phenotypes of *Fih1*$^{-/-}$ fibroblasts were HIF1α dependent or synergized with *Vhl* deficiency, it is unclear if FIH1 negatively regulates the HIF pathway in vivo. HIF1α activation can inhibit adipocyte differentiation in vitro (Yun et al., 2002), consistent with the reduced adipocyte density observed in *Fih1*$^{-/-}$ mice. On the other hand, HIFα activation in *Vhl*-deficient livers actually promotes hepatic steatosis (Rankin et al., 2009), in contrast to *Fih1*$^{-/-}$ mice. Therefore, further investigation is required to determine whether, as a general rule, FIH1 deficiency promotes metabolic phenotypes in vivo through HIFα activity.

### Mitochondria as Oxygen Sensors and PHD Regulators

Considerable evidence indicates that mitochondria also participate in $O_2$ sensing. Genetic and pharmacological approaches have been employed to inhibit components of the electron transport chain (ETC) in mitochondria. These studies have shown that in moderate hypoxia (1.5% $O_2$), mitochondria stimulate the production of cellular reactive oxygen species (ROS), which inhibit PHD activity and HIFα degradation (reviewed in Kaelin, 2005; Klimova and Chandel, 2008). These oxygen radicals emanate specifically from complex III of the ETC (Klimova and Chandel, 2008). Moreover, Waypa and colleagues were able to visualize redox changes within specific cellular compartments using a novel redox-sensitive fluorescent protein (RoGFP) (Waypa et al., 2010). Hypoxia-induced oxidants were observed in the inner-membrane space of mitochondria as well as in the cytosol, where ROS could influence PHD activity (Waypa et al., 2010). These findings support a model in which mitochondria sense $O_2$ deprivation and produce ROS to regulate PHD activity.

The role of mitochondria in $O_2$ sensing may be restricted, however, to moderate hypoxia (1.5%). As $O_2$ levels decline further to anoxia (0% $O_2$), HIFα can be stabilized in the absence of functional mitochondria, suggesting that factors in addition to mitochondrial ROS antagonize PHD activity in more severe $O_2$ deprivation (Kaelin, 2005; Klimova and Chandel, 2008). For example, this may represent a setting in which PHDs directly sense $O_2$ through its availability as a substrate.

In spite of the available data, the notion of mitochondria as $O_2$ sensors is controversial, and many questions remain unanswered. For instance, it is unclear what triggers mitochondria to release ROS in response to low intracellular $pO_2$ and whether ROS modulate PHD function directly or indirectly. Furthermore, it has been suggested that mitochondria signal to PHDs indirectly through their consumption of $O_2$ and not through ROS

production (Klimova and Chandel, 2008). This model could also explain the observation that mitochondrial inhibitors impair hypoxic stabilization of HIF1α: specifically, decreased mitochondrial $O_2$ consumption due to ETC inhibition could help maintain cytosolic $pO_2$ and, consequently, PHD activity. However, studies using cytochrome b mutant cells suggest this is not the case (Klimova and Chandel, 2008). In these cells, which generate mitochondrial ROS but fail to consume $O_2$, HIF1α can be stabilized by hypoxia in an oxidant-dependent manner. This indicates that mitochondrial ROS production, but not $O_2$ consumption, is important for HIFα stabilization.

Therefore, there may be multiple $O_2$ sensors—PHDs, FIH1, mitochondria, others—which collectively promote HIF molecular responses in hypoxia.

## The Growing Complexity of HIF Regulation

$O_2$ sensing via hydroxylases and mitochondria define a core feature of HIF regulation. However, the list of additional cues that modulate the HIF pathway is growing. These factors range from microRNAs to oncogenic signals, and some prominent examples are listed in Table 1. Two novel aspects of HIF regulation will be described below.

### *Sirtuins*

Sirtuins are a stress-responsive family of nicotinamide adenine dinucleotide ($NAD^+$)-dependent histone deacetylases that influence gene transcription, metabolism, DNA repair, and organism life span (Haigis and Sinclair, 2010). These enzymes respond to perturbations in the ratio of oxidized $NAD^+$/reduced NADH and, therefore, represent sensors of the cellular redox state (Denu, 2003). Sirtuins have recently been shown to modulate HIF activity, strengthening the link between cellular stress and HIF responses (Dioum et al., 2009; Lim et al., 2010; Zhong et al., 2010).

Garcia and colleagues showed that Sirt1 forms a complex with HIF2α, but not HIF1α, and deacetylates lysine residues in the HIF2α protein (Dioum et al., 2009). Sirt1 also co-occupies the *Epo* promoter with HIF2α and enhances HIF2α transcriptional activity in vitro. Finally, the Sirt1/HIF2α axis functions in vivo to regulate renal and hepatic erythropoietin expression. These data suggest that HIF2α-dependent EPO production is responsive to perturbations in the cellular redox state as well as changes in systemic $O_2$ availability (see HIF in a Systemic Response to Hypoxia). Sirt1 may not exclusively regulate HIF2α, however, as it was recently reported that Sirt1 interacts with and deacetylates HIF1α also (Lim et al., 2010). In contrast to HIF2α, HIF1α transcriptional activity is repressed by deacetylation. Under hypoxic stress, decreased cellular $NAD^+$ downregulates Sirt1, increases HIF1α acetylation, and thereby promotes the expression of HIF1α target genes. Interestingly, overexpression studies indicate that HIF2α can outcompete HIF1α for binding to Sirt1. Further in vivo experiments will be required to elucidate the complex role for Sirt1 in modulating both HIF1α and HIF2α activity, and to resolve certain discrepancies between these studies.

Sirt6 is also linked to HIF1α (Zhong et al., 2010). *Sirt6*$^{-/-}$ cells and mice display increased rates of glucose consumption and elevated expression of glycolytic genes, many of which are HIF1α targets. Sirt6 occupies the promoters of these genes and appears to enhance the deacetylation of associated histones, consistent with transcriptional repression. Furthermore, Sirt6 deficiency stimulates HIF1α protein expression. Using chemical inhibitors and RNA interference, the authors showed that HIF1α expression contributes, in part, to the metabolic phenotypes observed in *Sirt6*$^{-/-}$ cells and mice. Because the chemical inhibitors employed may lack target specificity, it will be important to determine if genetic loss of HIF1α also rescues phenotypes in *Sirt6*$^{-/-}$ animals. Lastly, Sirt6 and HIF1α appear to form a weak interaction, although the structure and functional significance of this complex requires further characterization. Based on these findings, Sirt6 appears to regulate glucose homeostasis by inhibiting HIF1α and glycolytic gene expression.

In sum, sirtuin activity can modulate HIF-dependent regulation of homeostatic processes such as erythropoiesis and glucose metabolism. The interplay between HIFs and sirtuins may also extend to stress settings such as hypoxic tumors, in which cellular redox balance is perturbed. However, the nature of the HIF response will depend on which sirtuin is engaged.

### *Intermittent Hypoxia*

Intermittent hypoxia (IH) occurs when tissue $O_2$ tension cycles between normal and hypoxic levels. This pattern of $O_2$ deprivation is particularly relevant during recurrent sleep apnea, in which transient pauses in breathing lead to chronic IH during sleep (Basner, 2007). Patients with this disease have a higher likelihood of developing hypertension, atherosclerosis, myocardial infarction, and stroke (Basner, 2007).

Prabhakar and colleagues have detailed how HIFα subunits are differentially regulated by IH. HIF1α protein expression is induced during IH, secondary to many factors including NADPH oxidase-dependent ROS generation (Peng et al., 2006; Yuan et al., 2008). Of note, this source of ROS is distinct from the mitochondrial ROS which inhibits PHD activity during continuous hypoxia. Studies in *Hif1α*$^{+/-}$ mice also demonstrated that HIF1α promotes the acute respiratory and cardiovascular consequences of IH. In contrast, HIF2α expression is repressed by IH through calpain-dependent mechanisms (Nanduri et al., 2009). HIF2α inhibition appears to contribute to IH-induced oxidative stress and cardiovascular responses in vivo. These data indicate that HIF1α and HIF2α may play opposing roles in IH-associated disease.

### *Differential Regulation of HIFα Subunits*

As the cases of IH and Sirt1 indicate, HIF1α and HIF2α can be regulated through distinct mechanisms. Additional examples from the literature support this concept. HIF1α, but not HIF2α, appears to be degraded in hypoxia in an Hsp70/CHIP-dependent fashion (Luo et al., 2009). On the other hand, *HIF2α* mRNA translation is uniquely responsive to iron content through an iron-response element in its 5′UTR (Sanchez et al., 2007). These observations underscore the complexity of the HIF response, and suggest that HIFα subunits require distinct forms of regulation because they mediate nonoverlapping biological effects, as was observed in mouse models of IH.

## The Role of HIF in Metabolism and Redox Homeostasis

In artherosclerotic diseases, tissues such as the heart, brain, and limb muscles are susceptible to ischemic insults (Beckman et al.,

**Table 1. Regulators of HIF Activity**

| | |
|---|---|
| HIF Regulators | |
| VHL | ↓ HIFα stability (see text) |
| PHD1/2/3 | ↓ HIFα stability (see text) |
| FIH1 | ↓ HIFα transcriptional activity (see text) |
| Siah1a/2 | ↑ HIF1α stability; promotes degradation of PHD1/3 in hypoxia (Simon, 2004) |
| RSUME | ↑ HIF1α stability; enhances SUMOylation (Carbia-Nagashima et al., 2007) |
| SENP1 | ↑ HIF1α stability; removes SUMO moieties (Cheng et al., 2007) |
| HSP90 | ↑ HIF1α stability (Isaacs et al., 2002) |
| COMMD1 | ↓ HIF1α stability and disrupts HIFα/β dimerization (van de Sluis et al., 2010, 2009) |
| HSP70/CHIP | ↓ HIF1α stability but not HIF2α stability ( Luo et al., 2009) |
| CITED2 | ↓ HIF1α activity (Bakker et al., 2007) |
| Metabolites/Related | |
| 2-oxoglutarate | ↓ HIFα stability as PHD cofactor (see text) |
| Ascorbate | ↓ HIFα stability as PHD cofactor (Kaelin and Ratcliffe, 2008) |
| Iron ($Fe^{2+}$) | ↓ HIFα stability as PHD cofactor (Kaelin and Ratcliffe, 2008) |
| IRP | ↓ *HIF2α* mRNA translation in response to high intracellular iron (see text) |
| NO | Modulates HIFα expression (Kaelin and Ratcliffe, 2008) |
| Intermittent hypoxia | ↑ HIF1α stability but ↓ HIF2α stability (see text) |
| Redox | |
| ROS | ↑ HIFα stability (see text); observed in inflammatory cells (Shatrov et al., 2003) |
| Sirt1 | ↓ HIF1α and ↑ HIF2α transcriptional activity (see text) |
| Sirt6 | Binds to and ↓ HIF1α stability/activity (see text) |
| MicroRNAs | |
| miR-107 | microRNA leads to ↓ ARNT expression (Yamakuchi et al., 2010) |
| miR-17-92 | miRNA cluster; microRNAs lead to ↓ HIF1α expression (Taguchi et al., 2008) |
| Oncogenes/Tumor Suppressors | |
| PI3K/Akt | ↑ HIF1α expression (Brugarolas and Kaelin, 2004; Mottet et al., 2003) |
| mTORC1 | ↑ *HIF1α* mRNA translation (Brugarolas and Kaelin, 2004; Bernardi et al., 2006) |
| GSK3β | ↓ HIF1α stability (Mottet et al., 2003) |
| p53 | ↓ HIF1α/ARNT expression (Blagosklonny et al., 1998; Yamakuchi et al., 2010; Sano et al., 2007; Schmid et al., 2004; Ravi et al., 2000) |
| β-catenin | Binds to HIF1α; ↑ HIF1α transcriptional activity (Kaidi et al., 2007) |
| Ras | ↑ HIF1α expression by ROS generation (Gerald et al., 2004) |
| ERβ | ↓ HIF1α stability (see text) |
| SD*H*/*FH* | Mutations in these genes lead to ↑ HIF1α stability (see text) |
| Inflammation | |
| NF- κB | ↑ *HIF1α* transcription (see text) |
| p44/42 MAPK | ↑ HIF1α expression downstream of LPS (Frede et al., 2006) |
| IFN-γ | ↑ HIF1α and HIF2α(?) expression (see text) |
| IL-4 | ↑ HIF1α and HIF2α(?) expression (see text) |

2002). Deprivation of $O_2$, nutrients, and growth factors causes stress in these pathologies, and cell death can ensue. The HIF pathway is activated and applies a critical adaptive response (Ratan et al., 2007; Shohet and Garcia, 2007). For instance, HIF1α expression correlates with increased preservation of brain and heart tissue in many (Ratan et al., 2007; Shohet and Garcia, 2007; Baranova et al., 2007) but not all (Helton et al., 2005) models of strokes and heart attacks, respectively.

### *HIF1α* and Glucose Catabolism

HIF1α is thought to mediate cardio- and neuroprotection, in part by reprogramming cellular metabolism. Because molecular $O_2$ serves as an electron acceptor in oxidative phosphorylation, a central adaptation to hypoxia is a shift toward nonoxidative forms of carbon metabolism and ATP production, such as anaerobic glycolysis (reviewed in Gordan et al., 2007b). HIF1α guides this shift by promoting the expression of glucose transporters, glycolytic enzymes, and *LDHA*, which replenishes $NAD^+$ for further glycolysis (Gordan et al., 2007b).

Moreover, studies by Semenza, Denko, and their colleagues showed that pyruvate dehydrogenase kinase 1—encoded by the HIF1α target gene *PDK1*—represses the flux of pyruvate into acetyl-CoA, diverting carbon away from mitochondria and suppressing $O_2$ consumption (reviewed in Simon, 2006). In HIF1α-deficient cells, $O_2$ deprivation leads to reduced ATP levels, elevated ROS, and apoptosis (Simon, 2006; Gordan et al., 2007b). The oxidant stress observed in hypoxic HIF1α mutant cells appears to be secondary to inappropriate acetyl-CoA generation and TCA cycle activity, such that forced PDK1 expression reduces ROS and promotes survival in hypoxia. These findings clearly demonstrate that a HIF1α-dependent metabolic shift promotes viability during hypoxic stress.

HIF1α activity was more recently shown to influence the pentose phosphate pathway or PPP (Zhao et al., 2010). The PPP converts glycolytic intermediates into ribose-5-phosphate (R5P), a substrate for nucleotide biosynthesis (Tong et al., 2009). In drug-resistant leukemia cells, HIF1α promotes the flux of glucose carbon through a nonoxidative arm of PPP relative to the oxidative arm (Zhao et al., 2010). These effects are critical for leukemia cell growth and survival. HIF1α, therefore, redirects the metabolism of glucose for use both as an energy source and as a building block for RNA and DNA synthesis, and these adaptations are likely important for facilitating cell growth and survival in hypoxic tumors.

Overall, HIF1α can directly reprogram the metabolic state in cells, and this response is important in hypoxic settings such as vascular disease and cancer.

### HIF2α Functions in Metabolism

Many of the metabolic genes described above are directly regulated by HIF1α but not HIF2α (Hu et al., 2003; Raval et al., 2005). Nevertheless, HIF2α also plays a critical role in metabolism. Semenza and colleagues demonstrated that both HIF1α and HIF2α can modulate the expression of *cytochrome c oxidase* isoforms so as to maximize efficiency of the ETC (reviewed in Gordan et al., 2007b). Defects in this response lead to impaired ATP production and elevated oxidant production in hypoxia.

HIF2α also has some unique targets in cellular redox homeostasis. Garcia and colleagues showed that HIF2α stimulates the expression of genes encoding antioxidant enzymes, such as *SOD2*, in mice (reviewed in Gordan et al., 2007b). This transcriptional program appears to suppress aberrant ROS accumulation, such that HIF2α deficiency leads to severe striated muscle damage. More recently, HIF2α has been demonstrated to play a similar function in renal cancer: HIF2α depletion leads to reduced expression of genes with anti-oxidant functions, such as *heme oxygenase 1* (*HMOX1*) and others (Bertout et al., 2009). In correlation, HIF2α loss induces increased cellular ROS, activation of p53, and tumor cell death. These effects are all enhanced with ionizing radiation treatment, which robustly elevates cellular ROS levels. Therefore, HIF2α promotes redox homeostasis and cellular viability in multiple settings. Moreover, HIF2α can mediate tumor cell resistance to ionizing radiation.

Studies in *Phd1*$^{-/-}$ mice provide further evidence that HIF2α can buffer tissues from hypoxic stress (Aragones et al., 2008). When placed under ischemic stress, *Phd1*$^{-/-}$ limb skeletal muscle is protected from oxidative damage and cell death. Interestingly, similar effects of PHD1 inhibition have been observed in liver ischemia (Schneider et al., 2010). The ischemic tolerance in *Phd1*$^{-/-}$ skeletal muscle is predominantly dependent on HIF2α, which is negatively regulated by PHD1. HIF2α may promote this tolerance by modulating glucose metabolism, for *Phd1*$^{-/-}$ muscle displays a metabolic shift toward anaerobic glycolysis and elevated expression of *Pdk4*. Like Pdk1, Pdk4 inhibits the mitochondrial consumption of glucose-derived carbon (Huang et al., 2002). Although HIF2α does not alter glucose utilization directly (Hu et al., 2003; Raval et al., 2005), it could function indirectly through regulation of PPARα, which is essential for ischemic tolerance in *Phd1*$^{-/-}$ skeletal muscle (Aragones et al., 2008) and may regulate *Pdk4* expression (Huang et al., 2002). Alternatively, HIF2α's more established role in redox homeostasis (reviewed in Gordan et al., 2007b; Bertout et al., 2009) could contribute to the ischemic phenotypes in *Phd1*$^{-/-}$ skeletal muscle.

Collectively, these observations indicate that both HIF1α and HIF2α control cell metabolism and redox homeostasis through nonoverlapping transcriptional programs (Figure 2).

### HIF Regulation of Lipid Metabolism

In tissues such as the heart and liver, lipids provide a rich source of energy via oxidative phosphorylation (Jungermann, 1988; Shohet and Garcia, 2007). In the setting of hypoxic stress, lipid metabolism is reprogrammed to suppress mitochondrial oxidation of lipid-derived carbon. Specifically, hypoxia stimulates lipid storage and inhibits lipid catabolism through β oxidation (Huss et al., 2001; Whitmer et al., 1978; Bostrom et al., 2006).

It was previously unclear if HIFs control these adaptations. However, a recent study implicates HIF2α in the regulation of lipid metabolism (Rankin et al., 2009). Mice with liver-specific deletion of *Vhl* exhibit fatty livers (or hepatic steatosis), marked by increased lipid droplet deposition and decreased fatty acid consumption. In mutant livers, genes involved in β oxidation are reduced, while those important in lipid storage are enhanced. These phenotypes are dependent on HIF2α more so than HIF1α. Therefore, in the pseudohypoxic context of *Vhl*-deficient livers, HIF2α represses lipid catabolism and oxidation (Figure 2).

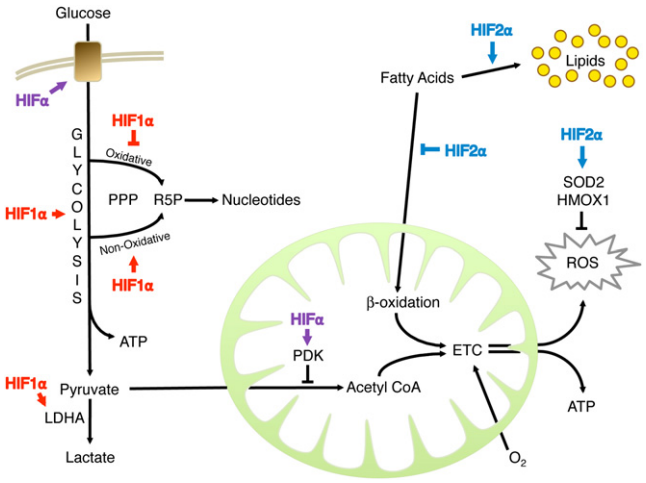


**Figure 2. HIFα Control of Cell Metabolism**

HIFs modulate cellular metabolism to facilitate cellular adaptation to low-oxygen environments. Glucose consumption and glycolysis are promoted primarily by HIF1α, while fatty acid storage is promoted by HIF2α. Both factors inhibit mitochondrial consumption and oxidation of carbon, leading to a decreased production of ATP through oxidative phosphorylation and less ROS as a byproduct. Instead, glycolysis makes a larger contribution to ATP synthesis in the cell. Furthermore, HIF2α inhibits ROS production through SOD2 and other targets.

## The Role of HIF in Vascular Responses to Hypoxia

It is well established that, in response to hypoxia, HIF1α and HIF2α regulate angiogenic genes such as *vascular endothelial growth factor* (Manalo et al., 2005; Hu et al., 2003; Kelly et al., 2003). In correlation, HIFs are known to play essential roles in embryonic vascular development (Ramirez-Bergeron et al., 2006; Covello and Simon, 2004). Recent studies have demonstrated how HIFs can influence the adult vascular system, specifically during angiogenesis in pathologic settings.

### HIF1α in Ischemia-Induced Angiogenesis

Growing evidence supports a role for HIF1α activity in the neoangiogenic response to tissue ischemia. In a femoral artery ligation model of hindlimb ischemia, ischemic limb muscle in *Hif1α*$^{+/-}$ mice exhibits impaired HIF1α induction, defective activation of angiogenic factors, and decreased reperfusion (Bosch-Marce et al., 2007). Conversely, adenoviral delivery of constitutively active HIF1α to the site of ligation leads to enhanced reperfusion. Therefore, HIF1α expression is essential and sufficient to promote reperfusion in ischemic skeletal muscle.

The authors also demonstrated that as mice age, the ischemic induction of HIF1α diminishes, and, in association, reperfusion is compromised. This is more than a correlation, for ectopic HIF1α

expression partially rescues limb perfusion in old mice. Interestingly, impaired HIF1α activation is also observed in the hypoxic skin wounds of aged diabetic mice (Liu et al., 2008). These findings underscore the importance of aging as a modulator of ischemic responses. This is especially crucial because peripheral arterial disease, the vascular disease which femoral artery ligation models, is associated with age (Beckman et al., 2002).

A proangiogenic role for HIF1α has also been described in other injury models such as hypertrophic cardiomyopathy, myocardial infarction, skin wound healing, and retinal neovascularization (Sano et al., 2007; Jiang et al., 2009; Yoshida et al., 2010; Heinl-Green et al., 2005; Mace et al., 2007; Liu et al., 2008; Botusan et al., 2008). Relative to HIF1α, the angiogenic functions of HIF2α have not been as extensively tested in these disease models (Dioum et al., 2008). In sum, these data suggest that HIFα expression, and in particular HIF1α expression, in ischemic tissues promotes angiogenesis. This has strong clinical implications for angiogenic therapies in patients with ischemic disease (see HIF-Targeted Therapies).

These findings also raise questions about the critical cell types in which HIFs operate during angiogenesis. While HIF activation in tissue parenchyma can influence EC behavior indirectly through expression of angiogenic factors, HIFs have also been shown to play essential roles within the vascular endothelium during vessel formation (see next section).

### *HIF in Endothelial Cells*

In ischemic tissues, ECs respond to cues provided by numerous distinct cell populations—including parenchyma, pericytes, and inflammatory cells—to form new blood vessels (Carmeliet, 2005). They also alter their function in direct response to changes in $O_2$ availability. For instance, hypoxia and HIF1α can direct ECs to form tube-like structures in vitro, mimicking morphologic changes which arise during angiogenesis (Yamakawa et al., 2003; Manalo et al., 2005).

Several studies have evaluated how HIFs regulate EC functions in hypoxic settings in vivo. This began with studies of mice with EC-specific deletion of *Hif1α* (Tang et al., 2004). Mutant mice exhibit defective blood vessel growth in hypoxic settings such as skin wounds and xenograft tumors. In correlation, isolated mutant ECs exhibit defective hypoxic activation of VEGF and its receptor VEGFR2 as well as impaired cell proliferation and migration. Therefore, the authors proposed that HIF1α promotes an autocrine VEGF/VEGFR2 loop in ECs that promote their functions in tissue angiogenesis.

HIF2α is highly expressed in ECs during development, suggesting it also may play a cell-autonomous role in this cell type (Ema et al., 1997). Mice with EC-specific deletion of *Hif2α* exhibit homeostatic defects in vessel integrity as well as impaired tumor angiogenesis (Skuli et al., 2009). Xenograft tumors in mutant mice are smaller, more hypoxic, and possess fewer luminized (functional) vessels relative to tumors in control mice. Mutant ECs from these animals exhibit defective adherence and impaired hypoxic induction of genes with functions in cell adhesion. These findings indicate that HIF2α instructs ECs to form more functional blood vessels, and this role is critical for tumor development.

Of note, *VEGF* expression is unaffected in HIF2α mutant ECs, implying that it is a HIF1α-specific target in ECs (Tang et al., 2004; Skuli et al., 2009; Manalo et al., 2005). This is consistent with prior studies indicating that HIF1α and HIF2α can promote the expression of distinct genes in ECs and, therefore, may carry out unique functions in this compartment (Elvert et al., 2003).

Overall, these studies highlight how ECs respond to local hypoxia during vessel growth and how HIFs mediate this response, particularly in tumor settings (see Figure 4C). Therefore, inhibiting HIF function in ECs could have utility in the treatment of cancer. This could be problematic, however, in situations where HIFs play tumor suppressive roles (see The Role of HIF in Cancer).

### *PHD2 in Endothelial Cells*

Studies in *Phd2*$^{+/-}$ mice indicate that PHD inhibition in ECs may have clinical implications (Mazzone et al., 2009). Xenograft tumors grown in *Phd2* heterozygotes are less hypoxic and have more functional vessels than those in control mice. ECs from these animals are more quiescent and exhibit an altered transcriptional program dependent on HIF2α. The authors propose that this transcriptional response directs ECs to form more organized vessels in tumors, and that this "normalization" plays a causal role in reducing the number of tumor metastases in mutant mice. It has also been suggested that normalization of tumor vessels can enhance tumor perfusion and potentially drug delivery (Jain, 2005). Therefore, PHD inhibition could be effective in several aspects of cancer therapy. However, the authors caution that in their models, PHD activity was impaired in the microenvironment but not in the tumor cells. This is an important distinction given the oncogenic role of HIFs (see next section).

## The Role of HIF in Cancer

There is ample evidence that solid tumors frequently encounter hypoxic stress. Rapidly proliferating cancer cells may outgrow their vascular network, limiting $O_2$ diffusion within the tumor. Hypoxic stress can also be caused by perfusion defects as a result of abnormal tumor blood vessel structure and function. Not surprisingly, therefore, solid tumors often exhibit high levels of HIFα accumulation (reviewed in Bertout et al., 2008). It should be noted that HIFα expression in cancer cells is also increased via hypoxia-independent mechanisms (see Table 1 and relevant sections). Genetic alterations such as *VHL* mutation in renal cell carcinoma, mutations in the Wnt/β-catenin signaling pathway in colon carcinoma, and other oncogenic events have been reported to result in HIFα stabilization (reviewed in Kaelin, 2008). Collectively, these findings indicate that HIFα expression and the downstream activation of the hypoxic stress response are widespread in many cancers.

Work from many laboratories has revealed that HIF-regulated gene responses play key roles in various aspects of cancer development, including proliferation (*MYC*), angiogenesis (*VEGF*, *PDGF*), apoptosis/autophagy (*NDRG2*, *BNIP3*), metabolism (*PDK1*, *LDHA*), DNA damage response (*GADD45A*), microRNAs (*MIR210*), extracellular matrix remodeling (*LOX*, *MMP1*), cell migration, and invasion (*CXCR4*, *SDF1*) (Huang et al., 2009; reviewed in Bertout et al., 2008; reviewed in Kaelin, 2008) (Figure 3). The importance of HIF activity in cancer is demonstrated by the fact that increased HIFα expression correlates with poor clinical prognosis in many cancer types (reviewed in Semenza, 2007).

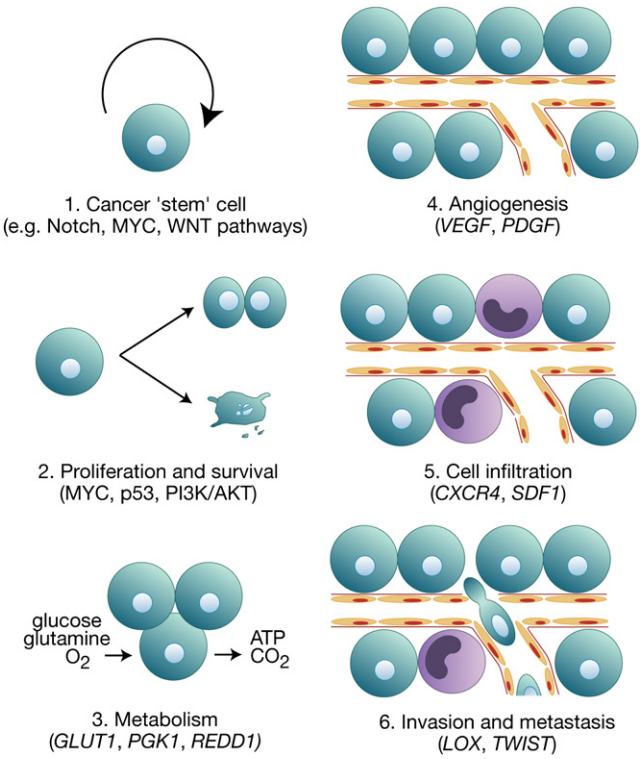


**Figure 3. Effects of HIF on Multiple Steps of Cancer Development**
HIF is stabilized by hypoxia and other nonhypoxic stimuli in many cancers. HIF activity in cancer has been associated with (1) putative cancer "stem" cell maintenance and increased expression of genes involved in (2) proliferation and survival, (3) metabolism, (4) angiogenesis, (5) recruitment of infiltrating cells such as TAMs and bone marrow-derived cells, and (6) tumor cell invasion and metastasis. Some examples of HIF-regulated genes and oncogenic pathways are given in parentheses.

### *HIFα Subunits and Tumorigenesis*

Surprisingly, HIF1α expression correlates with lower cancer stage or decreased patient mortality in certain cancers; examples include non-small-cell lung cancer, head and neck squamous cell carcinoma, and neuroblastoma (reviewed in Bertout et al., 2008). HIF2α expression in these malignancies, on the other hand, is a negative prognostic factor. This difference between HIF1α and HIF2α expression suggests that HIFα subunits may contribute differently toward tumorigenesis in certain cancers.

The distinct roles of HIF1α and HIF2α in tumorigenesis have been studied most thoroughly in *VHL*-deficient clear cell renal cell carcinoma (ccRCC). *VHL*-deficient ccRCC cluster into tumors which express either both HIF1α and HIF2α or HIF2α only (Gordan et al., 2008). Overexpression and knockdown studies of HIF1α and HIF2α in *VHL*-deficient ccRCC cell lines indicate that HIF2α, but not HIF1α, is necessary for tumor growth (reviewed in Kaelin, 2008). One possible explanation for this effect is that HIF1α antagonizes MYC function, whereas HIF2α promotes MYC activity (Gordan et al., 2007a). Microarray profiling of ccRCC specimens revealed that compared to tumors expressing both HIFα isoforms, tumors exclusively expressing HIF2α upregulate MYC target genes, proliferate faster, and are relatively resistant to replication stress (Gordan et al., 2008). Another mechanism by which HIFs exert opposing effects on tumor behavior lies in the hypoxic regulation of the tumor suppressor protein p53. HIF1α binds to p53, resulting in p53 stabilization and hypoxia-induced cell death (Moeller et al., 2005; An et al., 1998). This interaction between HIF1α and p53 is probably a late evolutionary development in higher organisms, as HIF1α indirectly inhibits the p53 homolog CEP-1 in *C. elegans* after radiation-induced DNA damage (Sendoel et al., 2010). In contrast, recent experiments have shown that HIF2α indirectly suppresses p53 activity and thereby promotes radioresistance and chemoresistance in tumor cells (Bertout et al., 2009; Roberts et al., 2009). These findings indicate that certain cancers, including but not limited to renal cell carcinoma, may differ in their tumor behavior and drug response according to the expression of HIF1α and/or HIF2α. Whether selective pressures exist in renal cell carcinomas for the loss of HIF1α and gain of HIF2α expression and whether HIFα expression patterns influence renal cancer progression remain subjects for further study.

Does HIF2α have greater oncogenic capacity than HIF1α in non-*VHL* malignancies? A recent report demonstrated that shRNA-mediated inhibition of HIF2α, but not HIF1α, in multiple human cancer cell lines reduced cell proliferation in vitro and subcutaneous xenograft growth in mice (Franovic et al., 2009). However, functional rescue experiments using exogenous HIF2α were lacking. In vivo models of lung tumorigenesis suggest a more complex role for HIF2α in cancer. Constitutively stabilized HIF2α increases lung tumor burden, tumor vascularity, and local invasion in *Kras* mutant mice (Kim et al., 2009). Intriguingly, a lung-specific deletion of HIF2α in the same *Kras* mutant model similarly enhances lung tumorigenesis (Mazumdar et al., 2010). A clue to this paradox may lie in the observation that HIF2α gain of function and HIF2α loss of function promote tumorigenesis via two unrelated mechanisms (Mazumdar et al., 2010; Kim et al., 2009). If we assume that different HIF2α targets have varying activation thresholds—due to HRE sequence conservation, coregulation by other transcription factors, composition of the transcription machinery, etc.—then according to the extent of HIF2α stabilization, either tumor suppressive (e.g., AKT inhibition) or tumor promoting effects (e.g., angiogenesis, epithelial to mesenchymal transition) may ensue.

The biology of HIF3α in relation to tumorigenesis remains largely unstudied. HIF3α is downregulated in renal cell carcinoma specimens, consistent with its known function as a dominant-negative inhibitor of HIF1α and HIF2α (Maynard et al., 2007). In summary, HIF1α, HIF2α, and HIF3α have varying effects on cancer development because of their context-dependent functions and distinct modes of action.

### *HIF and Metastasis*

Epithelial-to-mesenchymal transition (EMT) is a key feature of invasive cells and can be characterized by the loss of epithelial cell-cell contact and the acquisition of mesenchymal features and motility. Hypoxia and HIF influence the expression of many EMT regulators to promote metastasis. Studies by Maxwell and colleagues revealed that HIF1α expression in renal cell carcinoma is sufficient to induce the loss of E-cadherin and an increase in invasion (reviewed in Kaelin, 2008). HIF1α directly regulates *TWIST1* transcription and increases tumor cell

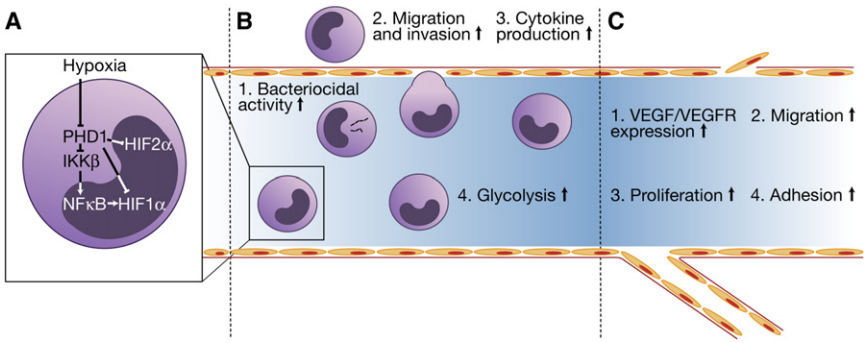


**Figure 4. Macrophage and Vascular Responses to HIF**

(A) NFκB-dependent regulation of HIF in macrophages. In addition to direct HIF stabilization, hypoxic inhibition of PHDs results in IKK-mediated degradation of the NFκB inhibitor IκB. Activated NFκB directly transactivates *HIF1α*.

(B) HIF activity is involved in multiple aspects of macrophage behavior via the induction of genes involved in (1) bacterial killing (*NOS2*, *CRAMP*), (2) migration and invasion (*CXCR4*, *FN1*, *MCSFR*), (3) cytokine production (*IL1β*, *IL6*, *IL12*, *TNFα*), and (4) metabolism (*GLUT1*, *PGK1*).

(C) HIF1α stabilization in ECs increases (1) VEGF expression, (2) migration, and (3) proliferation, whereas HIF2α stabilization promotes (4) EC adhesion to the extracellular matrix.

invasiveness and metastasis in head and neck squamous cell cancer (Yang et al., 2008). In prostate cancer, HIF1α promotes SNAIL1 nuclear localization in a VEGF-dependent manner (Mak et al., 2010). This finding is clinically relevant and implicates HIF1α expression in prostate cancer progression, because low-grade prostate tumors repress HIF1α via estrogen receptor β (ERβ) activity, while high-grade tumors downregulate ERβ, resulting in increased HIF1α expression, SNAIL1 nuclear localization, and metastasis (Mak et al., 2010). HIF1α also induces *lysyl oxidase* (*LOX*), which is an extracellular matrix remodeling enzyme as well as an upstream regulator of SNAIL1. As Giaccia and colleagues demonstrated, inhibition of LOX reduces tumor cell invasion, adhesion, and metastasis in an orthotopic breast cancer model (reviewed in Bertout et al., 2008). Recent work from the same group indicates that LOX secreted by the primary tumor remodels distant premetastatic sites to recruit tumor and stromal cells (Erler et al., 2009). The hypoxic tumor microenvironment therefore promotes metastasis via the activation of multiple HIF-responsive genes that together regulate all stages of cancer spread, including invasion, intravasation, and distant extravasation.

### HIF and Tumor Angiogenesis

HIF exerts similar effects on ECs in both tumor and nonmalignant tissues to mediate angiogenesis (see Figure 4C and relevant section). However, unlike "normal" blood vessels, tumor-associated vasculature is leaky, tortuous, and noncontiguous (reviewed in Jain, 2005). Tumor-associated endothelium interacts with tumor cells as well as nonmalignant stromal cells, such as fibroblasts and infiltrating bone marrow-derived cells. These cell types differ widely in their responses to hypoxic stress and therefore may contribute differently to tumor angiogenesis. For example, HIF activity in glioblastoma promotes tumor angiogenesis, as HIF1α inhibition in glioblastoma cells reduces vascular remodeling and normalizes tumor vasculature (Du et al., 2008). Paradoxically, HIF1α depletion in these cells also increases perivascular invasion, because of the direct effect of decreased VEGF levels on glioblastoma cell migration (Du et al., 2008). The development of tumor vasculature also appears to require myeloid-derived VEGF specifically. Deletion of the HIF target gene *Vegf* in myeloid cells increases murine mammary tumor growth, tumor oxygenation, and tumor sensitivity to chemotherapy, most likely due to "normalization" of tumor vessels (Stockmann et al., 2008). In contrast, haploinsufficiency of the HIF regulator *Phd2* in nonmalignant tissues allowed the "normalization" of xenograft tumor vasculature, improved oxygenation, and reduced metastasis (Mazzone et al., 2009). However, the dependence of these effects on HIF stabilization remains uncertain. These studies illustrate that the tumor vasculature responds to distinct and perhaps opposing HIF activities in different cell types. Therefore, selective manipulation of the hypoxic stress response in distinct tumor subcompartments may be more effective than systemic HIF inhibition as an antitumor strategy.

### HIF and Cancer Stem Cells

Hypoxia can promote an undifferentiated state in certain populations of stem and progenitor cells (Yoshida et al., 2009; Keith and Simon, 2007). Similarly, hypoxia and HIFs may contribute to the maintenance of putative cancer "stem" cells. HIF depletion in $CD133^+$ glioblastoma cells, which are enriched for cancer stem cells, reduces their tumorigenic and angiogenic potential in vitro and in vivo (Li et al., 2009). Furthermore, HIF2α is selectively expressed in the $CD133^+$ subpopulation of glioblastoma cells, whereas HIF1α expression is widespread among both tumorigenic and nontumorigenic cells, suggesting that HIF2α may fulfill a specific function in glioblastoma stem cells (Li et al., 2009). In a separate study, a small subset of immature cells in human neuroblastoma specimens was found to express neural crest markers and HIF2α; upon HIF2α knockdown, these cells underwent early sympathetic differentiation (Pietras et al., 2009). However, the precise identity and function of these cells remain unclear. It is interesting to note that both $CD133^+$ glioblastoma cells and putative neuroblastoma progenitor cells express high levels of HIF2α while residing in periendothelial niches (Pietras et al., 2009; Calabrese et al., 2007). Although the extent of $O_2$ saturation within these capillaries is unknown, these findings suggest that HIFα expression in certain cancer cell subpopulations may be controlled by both hypoxia and nonhypoxic stimuli, including metabolic aberrations in cancer.

### HIF Regulation by Cancer Metabolism

Inactivating homozygous mutations in the TCA cycle genes *fumarate hydratase* (*FH*) and *succinate dehyrogenase* (SD*H*) lead to elevated HIF1α expression in cells and human tumors (reviewed in King et al., 2006). Mice bearing kidney-specific inactivation of *Fh1* were generated and develop renal cysts marked by HIF activation (reviewed in Kaelin and Ratcliffe, 2008). HIF1α appears to be induced because of increased cellular levels of fumarate and succinate, which can inhibit PHD activity directly or indirectly through promotion of cellular ROS

(Sudarshan et al., 2009; Kaelin and Ratcliffe, 2008; Klimova and Chandel, 2008). These findings clearly demonstrate the extent to which metabolism influences HIFα expression in cancer. While it is assumed that HIF1α activation is promoting oncogenesis in these tumor settings, HIFs can be tumor suppressive in some contexts. The *Fh1* mutant mice will be a useful tool for testing this premise directly.

More recently, heterozygous mutations in isocitrate dehydrogenase 1/2 (*IDH1/2*) have been linked to cancer. Mutant IDH1 proteins are defective in their ability to oxidize isocitrate into α-ketoglutarate (αKG) (Dang et al., 2009; Zhao et al., 2009). Zhao and colleagues suggested that through a dominant-negative effect, cells expressing mutant IDH1 have reduced αKG levels, a substrate for PHDs. Therefore, HIF1α is indirectly stabilized. HIF1α expression is also elevated in human gliomas with mutant *IDH1*. Hence, they proposed that mutations in *IDH1* promote cancer by indirectly activating HIF1α, in the manner of *FH* and SD*H* mutations. On the other hand, multiple studies have since shown that lysates from *IDH1/2* wild-type and mutant cancers have comparable levels of αKG, indicating that one wild-type allele of *IDH1/2* may be sufficient to generate αKG in vivo (Dang et al., 2009; Ward et al., 2010; Gross et al., 2010). As a consequence, comparable levels of substrate should be available for PHD activity in wild-type and mutant cancers. This indicates the initial IDH1/HIF1α model may be inaccurate: *IDH1* mutations may stimulate oncogenic HIF responses, but not necessarily through decreased αKG levels.

Moreover, mutant IDH1 has an unexpected reverse activity which reduces αKG and generates 2-hydroxyglutarate (2HG), a metabolite associated with brain tumors (Aghili et al., 2009). Elevated 2HG is also observed in *IDH1/2* mutant glioblastoma and acute myeloid leukemia (Dang et al., 2009; Ward et al., 2010; Gross et al., 2010). Therefore, it has been suggested that this metabolite, synthesized by mutant IDH1/2, promotes cancers of the brain and blood. The molecular and cellular functions of 2HG, however, are poorly understood. One possibility is that 2HG inhibits PHD activity and, in turn, promotes oncogenic HIF responses in *IDH1/2* mutant tumors. 2HG may promote cancer through HIF-independent mechanisms as well. Further investigation is required to test the role of 2HG in the HIF pathway and, more generally, in tumor cell functions.

Overall, the latter studies on *IDH1/2* mutations underscore the notion that metabolic enzymes can be oncogenic as well as tumor suppressive (e.g., FH and SDH). They also challenge the concept that mutations in metabolic genes induce cancer solely through aberrant HIF responses.

## The Role of HIF in Inflammation

In response to inflammatory stimuli, local vascular permeability increases, resulting in the increased delivery of effector cells and nutrients to the inflamed tissue. The combination of reduced circulation at the site of inflammation and increased metabolic demand from infiltrating immune cells and pathogens eventually leads to the local depletion of $O_2$, resulting in hypoxia. Hypoxia and associated HIF activation have been observed in tissue specimens from patients with inflammatory conditions such as arthritis, artherosclerosis, and autoimmune diseases (reviewed in Nizet and Johnson, 2009).

### HIF Regulation in Inflammatory Cells

The response to hypoxic stress is tightly coupled to the immune response via NF-κB signaling (Figure 4A). Work from Nizet, Chilvers, Taylor, and their colleagues has demonstrated that HIF promotes NF-κB activity in macrophages, neutrophils, and nonimmune cells (reviewed in Nizet and Johnson, 2009). Hypoxia inhibits PHD1 activity and thereby IKK hydroxylation, resulting in IKK activation and phosphorylation of IκB. Subsequent IκB degradation liberates NF-κB from the cytoplasm, resulting in the transcription of downstream target genes, including inflammatory cytokines (Cummins et al., 2006). Interestingly, NF-κB signaling in activated macrophages directly regulates *Hif1α* transcription (Rius et al., 2008). Macrophages lacking IKKβ cannot stabilize HIF1α after hypoxic or microbial challenge and exhibit decreased HIF target gene expression. However, activation of NF-κB alone is insufficient for HIF1α stabilization, indicating that maximal HIF1α accumulation depends on both transcriptional regulation by NF-κB and post-translational regulation by hypoxia (Rius et al., 2008). This coordinated response suggests a mechanism for graded HIF1α expression in immune cells, in which maximal HIF1α activity is induced in cells located at the site of most severe inflammation, where hypoxic and inflammatory stresses are simultaneously present.

In contrast, hypoxic induction of HIF2α does not require IKKβ (Rius et al., 2008). A recent report proposes that HIF1α and HIF2α are differentially stabilized in macrophages exposed, respectively, to $T_H1$ cytokines such as IFN-γ and to $T_H2$ cytokines such as IL-4 (Takeda et al., 2010). Because IFN-γ, but not IL-4, activates the NF-κB pathway in macrophages (Thieu et al., 2007), this finding suggests that HIF1α expression, but not that of HIF2α, is dependent on NF-κB. However, a subsequent study showed significant HIF2α induction with $T_H1$ cytokines IFN-γ and LPS (Imtiyaz et al., 2010). Available data on this aspect of HIF2α regulation in macrophages are therefore conflicting. Given the role of HIF-2α in myeloid-mediated inflammatory responses (see below), it will be important to fully assess the contribution of NF-κB signaling toward HIF2α activity in immune settings, as well as other mechanistic differences between HIF1α and HIF2α stabilization in hypoxic and inflammatory stress.

### HIF and Myeloid Cell Function

Myeloid cells—neutrophils, macrophages, and dentritic cells—play key effector roles in acute and chronic inflammation. Conditional inactivation of HIF1α or HIF2α in these cells has revealed crucial roles for both subunits in mediating inflammatory responses (Figure 4B). The absence of HIF1α in myeloid cells significantly weakens the inflammatory response in mouse models of arthritis and chronic dermatitis, and confers increased protection against LPS-induced sepsis (Peyssonnaux et al., 2007; Cramer et al., 2003). Macrophages lacking HIF1α exhibit decreased motility, invasiveness, and bacteriocidal activity (Peyssonnaux et al., 2005; Cramer et al., 2003). Similarly, conditional HIF2α loss in myeloid cells decreases macrophage motility and invasion and reduces the severity of cutaneous inflammation and LPS-induced endotoxemia (Imtiyaz et al., 2010). The cytokine response to LPS-induced sepsis, characterized by the production of TNF-α, IL-1β, IL-6, and IL-12, is reduced in the absence of either HIF1α or HIF2α (Imtiyaz et al., 2010;

Peyssonnaux et al., 2007). Interestingly, HIF1α and HIF2α coordinate similar inflammatory responses via distinct mechanisms. HIF1α controls the metabolic shift from oxidative phosphorylation to glycolysis in activated macrophages (Cramer et al., 2003). In contrast, HIF2α regulates the transcription of cytokines, such as *IL1β*, *IL12*, and *TNFα*, as well as genes involved in macrophage migration and chemotaxis, such as *FN1* and *CXCR4* (Imtiyaz et al., 2010). A gene expression profile analysis of primary human macrophages in which HIF1α or HIF2α were depleted confirms that HIF1α and HIF2α induce overlapping but distinct sets of genes (Fang et al., 2009).

Hypoxia and HIFs regulate other myeloid lineages also. Neutrophils express HIF1α, but not HIF2α, under hypoxic and inflammatory stress (Imtiyaz et al., 2010). HIF1α expression in these cells is required for host pathogen defense and neutrophil survival (Walmsley et al., 2006; Peyssonnaux et al., 2005). HIF1α expression in dentritic cells is required for the expression of costimulatory molecules after LPS exposure and the induction of allogenic T cell proliferation (Jantsch et al., 2008). In mast cells, HIF1α expression regulates the production of proinflammatory cytokines, vasodilators such as VEGF, and the histamine synthesizer histidine decarboxylase (reviewed in Nizet and Johnson, 2009). Therefore, HIFs coordinate the behavior of different immune cells in a hypoxic inflammatory milieu to produce a unified immune response.

### *HIF and Tumor-Associated Macrophages*

Studies in many cancer types have shown that macrophage infiltration correlates with unfavorable clinical prognosis (reviewed in Lewis and Pollard, 2006). Macrophages are recruited to tumor areas primarily by the production of chemoattractants by hypoxic tumor and stromal cells, such as the HIF target genes *CSF1* and *VEGF* (reviewed in Murdoch et al., 2004). Recent work has also shown that apoptotic cells, such as those in hypoxic regions of a tumor, produce soluble factors such as TGF-β to attract monocytes and macrophages (Herr et al., 2009). Once recruited, tumor-associated macrophages (TAMs) exhibit a highly dynamic immune phenotype which promotes tumor growth, angiogenesis, metastasis, and tumor immunosuppression. Furthermore, as Harris and colleagues noted, TAMs exhibit elevated HIF1α and HIF2α expression due to the hypoxic tumor microenvironment (reviewed in Murdoch et al., 2004).

HIF2α expression in breast and cervical cancer TAMs is correlated with unfavorable prognoses, suggesting a functional relevance for HIF2α in this setting (Kawanaka et al., 2008; Leek et al., 2002). Conditional HIF2α deletion in the myeloid lineage in mice has revealed key roles for HIF2α and TAMs in tumorigenesis. In mouse models of hepatocellular carcinoma and colitis-associated colon carcinoma, mice lacking HIF2α in their myeloid cells exhibit decreased recruitment of TAMs into tumor areas (Imtiyaz et al., 2010). This finding correlates with reduced tumor mitotic index, lower tumor grade, and a downward trend in the number and size of colitis-induced colon carcinomas (Imtiyaz et al., 2010). It will be of interest to explore whether HIF1α expression in TAMs is functionally relevant to tumor progression in similar in vivo models, and if so, whether HIF1α and HIF2α complement each other in this context. Recent work suggests that the absence of HIF1α in macrophages has no effect on tumor spheroid infiltration, tumor cell proliferation, or tumor invasiveness in vitro, but reduces cell death in tumor spheroid cultures (Werno et al., 2010).

## HIF in a Systemic Response to Hypoxia

Organs involved in erythropoiesis (red blood cell production) can respond to systemic hypoxia to increase red blood cell numbers and the $O_2$-carrying capacity of blood (reviewed in Fandrey, 2004). *EPO*, a preferential HIF2α target, stimulates this process (reviewed in Lee, 2008; Fandrey, 2004). Abundant data suggest that the PHD2/pVHL/HIF2α axis controls EPO levels and, therefore, adult erythropoiesis in humans and mice. Human genetic studies of familial polycythemia (abnormally elevated hemoglobin or red blood cell count) identified mutations in *VHL*, which impaired HIF1α degradation (Lee, 2008). Subsequent genetic and biochemical analyses have identified inactivating mutations in *PHD2* and activating lesions in *EPAS1*/*HIF2α* (Lee, 2008; Furlow et al., 2009). Mouse models bearing mutations in these genes have also been generated and exhibit abnormal erythropoiesis (Lee, 2008). More recent findings indicate that while the kidney and liver are the main organs producing EPO, HIFα expression in other distant tissues—skin and glial cells—also influences EPO production in response to hypoxia (Boutin et al., 2008; Weidemann et al., 2009).

Furthermore, studies comparing Tibetan highlanders and the closely related lowland Han Chinese have provided an evolutionary link between the PHD2/pVHL/HIF2α axis and erythropoiesis (Yi et al., 2010; Beall et al., 2010; Simonson et al., 2010). The authors compared the frequencies of single-nucleotide polymorphism (SNP) alleles between these groups, and noted significant divergence in allelic frequency of SNPs located in or near the *PHD2*/*EGLN1* and *EPAS1*/*HIF2α* genes. These findings correlated with lower hemoglobin and erythrocyte levels in the blood of Tibetan subjects, suggesting that the *PHD2* and *HIF2α* alleles common to Tibetan highlanders cause relatively decreased erythropoiesis. It has been proposed that the divergence in *HIF2α* and *PHD2* occurred through natural selection, whereby Tibetan *HIF2α* and *PHD2* alleles facilitate survival in the high altitude of the Tibetan plateau. For instance, Tibetans are resistant to developing chronic mountain sickness, which is marked by elevated erythropoiesis. These reports highlight the evolutionary implications of PHD2 and HIF2α function in red blood cell production.

In sum, studies in humans and rodents have contributed to a body of knowledge linking the PHD/pVHL/HIF pathway to erythropoiesis. This information is now being applied to the treatment of anemia (see below).

## HIF-Independent Responses to Hypoxic Stress

While this review emphasizes the role of HIFs in response to $O_2$ deprivation, the hypoxic response is an integration of multiple $O_2$-sensing pathways. Substantial evidence indicates that mTOR signaling and the UPR play critical roles in hypoxic adaptations by modulating protein translation, cell metabolism, and cell fate (Wouters and Koritzinsky, 2008; Sengupta et al., 2010; Spriggs et al., 2010; Buchberger et al., 2010). We must also consider that other transcriptional regulators, such as PGC1α, can complement the HIF response in ischemic

settings: PGC1α, independent of HIF, promotes VEGF expression and neoangiogenesis in a model of hindlimb ischemia (Arany et al., 2008).

In addition, PHDs and FIH1 have non-HIFα substrates which may underlie some of their biological functions (Webb et al., 2009a). For example, recent reports indicate that prolyl hydroxylases play significant HIF-independent roles in cancer. PHD2 suppresses growth of xenograft tumors in a HIF- and, surprisingly, hydroxylase-independent fashion (Chan et al., 2009). PHD1/EglN2, on the other hand, promotes tumor growth through HIF-independent regulation of Cyclin D1 (Zhang et al., 2009). It is also important to note that all 2-oxoglutarate-dependent dioxygenases, including but not limited to PHDs, require $O_2$ for their enzymatic activity and therefore could potentially mediate HIF-independent responses to hypoxia.

These observations emphasize that hypoxic adaptations are mediated by more than the HIF response. Hypoxia can activate many distinct pathways and influence less-established branches of the canonical PHD/pVHL/HIFα pathway.

## HIF-Targeted Therapies

Many insights from HIF biology are being translated into clinical applications and, in particular, drug discovery. The most advanced HIF pathway-targeted pharmaceuticals in terms of clinical development, to date, are PHD inhibitors. These compounds, FG-2216 and FG-4592, are being evaluated for treatment of anemia and are currently in phase I and II clinical trials (http://clinicaltrials.gov/ identifiers NCT00456053, NCT00761657, NCT00978198, and NCT00978198).

### *HIF Activators*

In addition to PHD inhibition, several strategies to promote HIF1α activity and angiogenesis are in development for use in ischemic disease. In models of hindlimb ischemia, adenoviral delivery of constitutively active HIF1α has shown promise when administered alone or in combination with bone marrow-derived angiogenic cells (Bosch-Marce et al., 2007; Rey et al., 2009). HIF1α adenoviral therapy has also shown benefit in limb ischemia models in aged and diabetic mice (Bosch-Marce et al., 2007; Sarkar et al., 2009). These findings are significant if one considers that two large patient populations afflicted by atherosclerosis and associated ischemic diseases are diabetics and the elderly (Beckman et al., 2002).

Similar approaches with hybrid HIF1α/VP16 have been used in rabbit and diabetic rat models of limb ischemia and progressed through phase I and II clinical studies in patients with severe peripheral arterial disease (Vincent et al., 2000; Rajagopalan et al., 2007; Kajiwara et al., 2009). These interventions have also been applied to other ischemic injuries such as wound healing and myocardial infarction (Liu et al., 2008; Mace et al., 2007; Heinl-Green et al., 2005). In addition to gene therapy, PHD inhibitors have also shown utility in wound healing in diabetic animals (Botusan et al., 2008).

Overall, these findings indicate that HIF activating therapies are effective in preclinical studies of ischemia and merit investigation in patients suffering from ischemic disease.

### *HIF Inhibitors*

Transcription factors have historically been considered undruggable targets. However, interest in HIF inhibition as a therapeutic strategy remains high (Semenza, 2007). A high-throughput screen of FDA-approved drugs for anti-HIF activity revealed that digoxin and other cardiac glycosides inhibit *HIFα* translation and subcutaneous xenograft growth (Zhang et al., 2008). Importantly, HIFα inhibition is independent of digoxin's known effect on the $Na^+/K^+$ ATPase but necessary for its tumor suppressive effect (Zhang et al., 2008). Other HIF inhibitors identified in similar screens include the antiseptic dye acriflavine, and anthracyclines such as doxorubicin and daunorubicin (Lee et al., 2009a, 2009b). Screening for HIF inhibitors among approved agents means that identified compounds, although pharmacologically well studied and suitable for human use, can be presumed to exhibit HIF-independent effects stemming from their original therapeutic purposes. Therefore, for these drugs to be used in targeted HIF therapy, it will be crucial to demonstrate that HIF repression is sufficient for their intended biological effects.

Another challenge in HIF targeting involves the overlapping but distinct biological roles of HIFα subunits. Compounds that promote the binding of IRP1 to the 5′UTR of *HIF2α* mRNA decrease HIF2α hypoxic induction, but also repress HIF1α synthesis via an independent mechanism (Zimmer et al., 2008). An RNA antagonist of HIF1α, EZN-2968, reduces HIF1α protein and target gene expression in vitro and in vivo, but not that of HIF2α, and is being evaluated in a phase I clinical trial (Patnaik et al., 2009; Greenberger et al., 2008). We expect that RNAi will play a key role in targeted HIF therapy once effective and selective delivery methods become available.

With the emerging view of the importance of HIF1α and HIF2α in disease, there is a largely unmet need for specific HIF inhibitors. While combined inhibition of HIFα isoforms will be appropriate in certain disease situations, HIF1α- or HIF2α-specific therapies may be preferable in other scenarios.

### *Synthetic Lethality with VHL Deficiency*

In many cancers, reactivating tumor suppressors could provide a therapeutic benefit. However, it is challenging to design drugs for this purpose. Several groups, therefore, have adopted a synthetic lethal screening approach to overcome this hurdle. Two genes are synthetically lethal if inhibition of either gene is compatible with viability, but inhibition of both leads to cell death. In ccRCC, several groups have screened for genes which are synthetically lethal with *VHL* (Bommi-Reddy et al., 2008; Turcotte et al., 2008). One group screened a shRNA library targeting various kinases and identified several targets, such as CDK6, for which chemical inhibitors already exist (Bommi-Reddy et al., 2008). Another screen employed a drug library and identified a compound, STF-62247, which promoted autophagic cell death in *VHL*-deficient cells (Turcotte et al., 2008). Surprisingly, in several cases, *VHL*-replete cells with stable HIF2α expression were viable in the presence of drug- or kinase-specific shRNA, suggesting the observed death in *VHL*-deficient cells is HIF independent (Turcotte et al., 2008; Bommi-Reddy et al., 2008). In sum, these screens implicate new molecular targets and compounds in the treatment of *VHL*-deficient ccRCC.

## Conclusion

Hypoxic stress is characteristic of many pathological settings, and the HIFs direct critical adaptations to enable cells, tissues, and organisms to survive and thrive in these conditions. Recent

work has revealed new mechanisms of HIF induction, including PHD-dependent and -independent modes of regulation, as well as new effects of activating the HIF response in development and disease. In some cases, these responses promote disease progression, while in others, HIF responses are a part of disease recovery. Recent evidence has also highlighted the common and distinguishing features between HIF1α- and HIF2α-mediated responses in cancer, tissue ischemia, and inflammatory disease. A deeper understanding of how HIFα isoforms are uniquely regulated and how they can be selectively modulated will be essential for translating our current knowledge of the HIF pathway to clinical settings.

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